Oxalate Production by *Sclerotinia sclerotiorum* Deregulates Guard Cells during Infection[1][w]

Rejane L. Guimarães and Henrik U. Stotz*

Department of Horticulture, Oregon State University, Corvallis, Oregon 97331

Oxalic acid is a virulence factor of several phytopathogenic fungi, including *Sclerotinia sclerotiorum* (Lib.) de Bary, but the detailed mechanisms by which oxalic acid affects host cells and tissues are not understood. We tested the hypothesis that oxalate induces foliar wilting during fungal infection by manipulating guard cells. Unlike uninfected leaves, stomatal pores of *Vicia faba* leaves infected with *S. sclerotiorum* are open at night. This cellular response appears to be dependent on oxalic acid because stomatal pores are partially closed when leaves are infected with an oxalate-deficient mutant of *S. sclerotiorum*. In contrast to oxalate-deficient *S. sclerotiorum*, wild-type fungus causes an increase in stomatal conductance and transpiration as well as a decrease in plant biomass. Green fluorescent protein-tagged *S. sclerotiorum* emerges through open stomata from the uninfected abaxial leaf surface for secondary colonization. Exogenous application of oxalic acid to the detached abaxial epidermis of *V. faba* leaves induces stomatal opening. Guard cells treated with oxalic acid accumulate potassium and break down starch, both of which are known to contribute to stomatal opening. Oxalate interferes with abscisic acid (ABA)-induced stomatal closure. The Arabidopsis (*Arabidopsis thaliana*) L. Heynh. mutants *abi1*, *abi3*, *abi4*, and *aba2* are more susceptible to oxalate-deficient *S. sclerotiorum* than wild-type plants, suggesting that Sclerotinia resistance is dependent on ABA. We conclude that oxalate acts via (1) accumulation of osmotically active molecules to induce stomatal opening and (2) inhibition of ABA-induced stomatal closure.

Oxalic acid (ethanedioic acid) occurs ubiquitously in nature, sometimes as a free acid, but more commonly as soluble potassium or sodium oxalate or as insoluble calcium oxalate. Biosynthesis of oxalate occurs in members of all five kingdoms. Oxalate is associated with metabolic disorders and infectious diseases (Holmes and Assimos, 1998; Nakagawa et al., 1999). Several phytopathogenic fungi, including *Sclerotinia sclerotiorum* (Lib.) de Bary, produce millimolar concentrations of oxalate in infected tissues (de Bary, 1886; Ferrar and Walker, 1993). Oxalate is an essential virulence factor of *S. sclerotiorum* because mutants, which are deficient in oxalate biosynthesis, are less pathogenic than wild-type fungus (Godoy et al., 1990). In contrast to wild-type fungus, oxalate-deficient *S. sclerotiorum* is unable to produce oxalate during infection of petals, which are an important source of inoculum in the field and during in vitro cultivation (Godoy et al., 1990; Jamaux et al., 1995).

Enzymes that catabolize oxalate protect plants from Sclerotinia infection when their genes are expressed in stably transformed plants. Constitutive expression of wheat oxalate oxidase, an enzyme that converts oxalate into H_2O_2 and CO_2, enhances resistance of soybean (*Glycine max*; Donaldson et al., 2001) and sunflower (*Helianthus annuus*) plants (Burke and Riesenberg, 2003) to *S. sclerotiorum*. Similarly, overexpression of oxalate decarboxylase from the fungus *Collybia velutipes* protects tobacco (*Nicotiana tabacum*) and tomato against *S. sclerotiorum* (Kesarwani et al., 2000). Thus, oxalate metabolism has a profound influence on interactions between *S. sclerotiorum* and its hosts. This fungus infects more than 400 plant species and causes major economic losses of crops, such as sunflower, canola, soybean, peanut, bean, and broccoli, worldwide.

The precise mechanism of oxalate action during infection is not completely understood. However, oxalate has been proposed to remove calcium ions bound to pectins, which exposes host cell walls to catabolic enzymes of fungal origin (Bateman and Beer, 1965). Oxalic acid also favors plant cell wall degradation by shifting the pH of infected plant tissues close to the optimum of cell wall-degrading enzymes, such as polygalacturonase (Bateman and Beer, 1965). In addition, oxalate suppresses the defense-related oxidative burst of soybean and tobacco cells (Cessna et al., 2000). Conversely, constitutive expression of oxalate-degrading enzymes in plants increases defense gene induction (Kesarwani et al., 2000; Hu et al., 2003). These recent results suggest that oxalate impinges on plant signaling.

Oxalic acid causes wilting symptoms in Sclerotinia-infected plants (Noyes and Hancock, 1981; Kolkman and Kelly, 2000). In this study, we test the hypothesis that oxalate causes foliar dehydration by disturbing guard cell function. We provide evidence that oxalate alters guard cell osmoregulation and interferes with abscisic acid (ABA)-induced stomatal closure.

[1] This work was supported by the U.S. Department of Agriculture Sclerotinia Initiative Special Grants program (grant no. 58–5442–2–257) and by the Department of Horticulture at Oregon State University.

* Corresponding author; e-mail stotzhe@science.oregonstate.edu; fax 541–737–3479.

[w] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.049650.

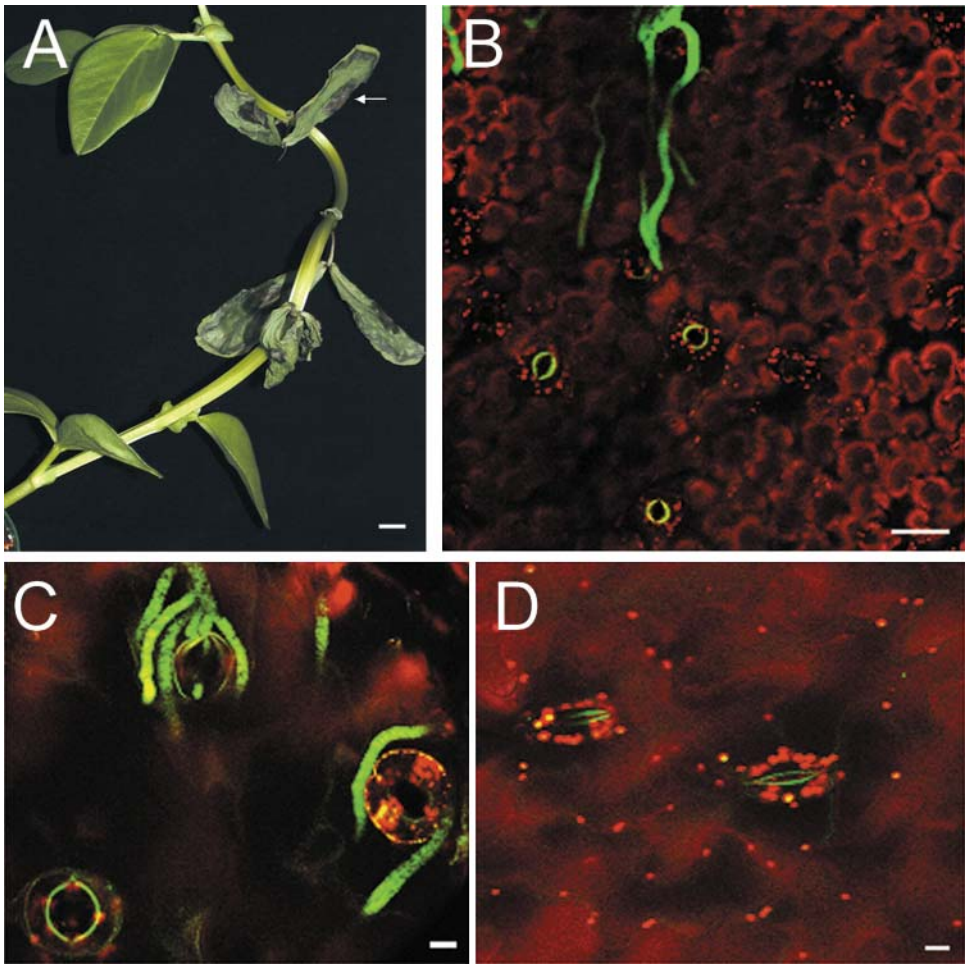

Figure 1. *S. sclerotiorum* causes wilting and prevents stomatal closure at night. A, Wilting symptoms of *V. faba*, leaves of which were infected with *S. sclerotiorum*. This plant was transferred from high- to low-humidity conditions 2 dpi to demonstrate foliar wilting. Arrow indicates a necrotic lesion. Unchallenged leaves above and below infected leaves do not show wilting symptoms. B to D, Confocal images of leaves, which were infected with a GFP-tagged strain (B and C) of *S. sclerotiorum* or unchallenged (D), were taken at night. B, *S. sclerotiorum* prevents stomatal closure in front of hyphae 20 hpi. C, Hyphae surround and exit open stomata 40 hpi. Bars = 1 cm (A), 50 μm (B), and 10 μm (C and D).

RESULTS

S. sclerotiorum Induces Oxalate-Dependent Wilting Symptoms by Deregulating Guard Cells

We used a green fluorescent protein (GFP)-tagged strain of *S. sclerotiorum* in conjunction with confocal microscopy to determine whether wilting symptoms (Fig. 1A) are the result of stomatal dysfunction during infection of *Vicia faba* leaves. *S. sclerotiorum* prevented closure of stomata in the dark (Fig. 1, B–D). The fungus exploited open stomatal pores to emerge from the uninoculated abaxial leaf surface (Fig. 1C; Supplemental

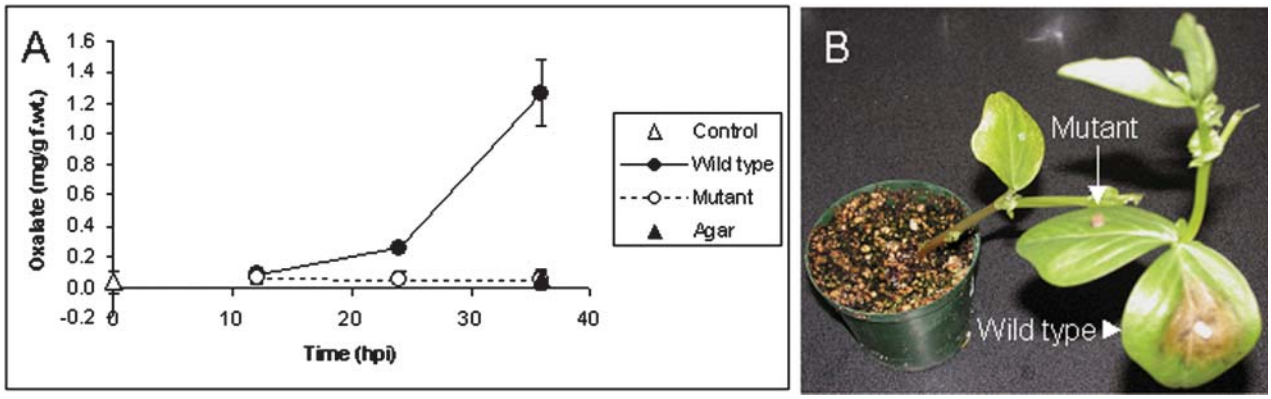

Figure 2. Effects of oxalate deficiency on pathogenicity of *S. sclerotiorum*. A, Oxalate production in leaves of *V. faba* plants infected with wild-type strain 1980 (●) or oxalate-deficient mutant A4 (○; Godoy et al., 1990). A leaf was removed from each plant prior to the onset of inoculation to determine endogenous oxalate levels (△). A mock-inoculated leaf was removed at the end of the experiment to test whether oxalate levels were altered during the course of the experiment (▲). Means and SD of three plants are shown. B, Image of a plant 2 dpi. Compared to the wild-type strain, oxalate-deficient *S. sclerotiorum* was much less virulent.

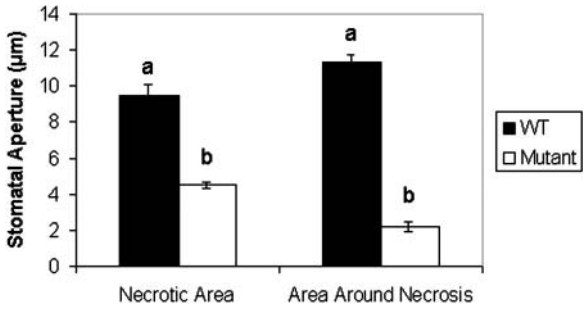

Figure 3. Infection-related changes in stomatal aperture are dependent on oxalic acid. Leaves of *V. faba* plants were infected with a wild-type or an oxalate-deficient mutant strain of *S. sclerotiorum*. Stomatal apertures were measured 3 dpi. Means ± SE are shown ($n \geq 32$). Letters a and b indicate significant differences ($P < 0.05$) according to ANOVA and Duncan's Multiple Range Test. This experiment was repeated three times with similar results.

Fig. 1, available at www.plantphysiol.org). Based on microscopic analysis of four leaves from two plants 2 d postinoculation (dpi), 22 ± 1 hyphae protruded through stomata, whereas 7 ± 1 hyphae penetrated through the cuticle (paired *t* test; $n = 118$; $P = 0.0008$). Stomata were open in advance of fungal colonization (Fig. 1B). All of the stomatal pores in the vicinity of hyphal growth were classified as open (≥5 μm; $n = 50$), whereas unchallenged leaves contained exclusively closed stomata (≤5 μm; $n = 50$).

To determine whether stomatal dysfunction depends on the production of oxalic acid, we compared stomatal apertures after infection of *V. faba* leaves with wild-type or oxalate-deficient *S. sclerotiorum*. Oxalate deficiency was confirmed by comparing oxalate accumulation in leaves infected with wild-type or mutant fungus (Fig. 2A). In contrast to wild-type *S. sclerotiorum*, oxalate levels in leaves infected with mutant fungus were not significantly different from control leaves. Two types of control leaves were used: (1) leaves removed prior to the onset of the experiment and (2) leaves mock-inoculated with agar and harvested 2 dpi. Oxalate concentrations increased 38-fold in leaves infected with wild-type *S. sclerotiorum* 2 dpi. Unlike wild-type *S. sclerotiorum*, which caused soft rotting lesions, mutant fungus caused a dry necrosis (Fig. 2B). These results are consistent with published data (Godoy et al., 1990) and suggest that differences in stomatal responses are likely due to defects in fungal oxalate biosynthesis. The oxalate-deficient mutant was less virulent than the wild-type fungus (Fig. 2B). Changes in stomatal aperture were detected within and around necrotic lesions. Open stomata occurred 5 mm beyond the necrosis in leaves infected with wild-type *S. sclerotiorum*, but only 0.8 mm beyond the necrosis in leaves infected with oxalate-deficient fungus. Disease susceptibility and stomatal aperture were closely correlated. Stomatal apertures of leaves infected with wild-type fungus were twice as wide in necrotic areas and five times wider in areas surrounding necroses when compared to leaves infected with the mutant fungus (Fig. 3).

We determined the effect of oxalate-dependent stomatal dysfunction on whole-plant physiology. Necrotic lesions advanced four times faster in stems of *V. faba* plants infected with wild-type *S. sclerotiorum* relative to the oxalate-deficient mutant fungus (Table I). Stomatal conductance and transpiration rates were significantly higher in plants infected with wild-type fungus than in plants challenged with the oxalate-deficient mutant or mock-inoculated plants. In addition, wild-type *S. sclerotiorum* significantly reduced stem fresh weight and dry weight over a period of 12 d when compared to the oxalate-deficient mutant or mock-inoculated controls. These data suggest that *S. sclerotiorum* manipulates stomata to increase water stress and pierce through the abaxial leaf surface.

Oxalate Induces Stomatal Opening in Detached Leaf Epidermis

We treated the abaxial epidermis of *V. faba* leaves with oxalic acid to characterize guard cell behavior. Oxalate induced maximal stomatal opening at a concentration of 1 and 10 mM oxalate at pH 3 and 6.3, respectively (Fig. 4A). Because the pK_a values of oxalic acid are 1.2 and 4.2, the majority of this acid is expected to exist as monodissociated and completely dissociated forms at pH 3 and 6.3, respectively. The aperture of stomatal pores increased with time (Fig. 4B). Concentrations of 1 to 10 mM oxalate significantly increased stomatal opening relative to the buffer control at pH 6.1 over a period of 3 h. Fluorescein diacetate staining indicated that loss of cell viability at 10 mM oxalate, pH 3, and at 100 mM oxalate, pH 6.3, caused a decline in stomatal aperture (Fig. 4). No reduction in cell viability was observed at ≤1 and ≤10 mM oxalate at pH 3 and 6.3, respectively.

Table I. *Physiological changes during S. sclerotiorum infection*

LS, Lesion size; *g*s, stomatal conductance; E, transpiration; FW, fresh weight; DW, dry weight. Values are means ± SEM of 20 leaves (*g*s, E) and five stems (LS, FW, DW) from three independent experiments. Letters a, b, and c represent significant differences ($P < 0.05$) according to Duncan's Multiple Range Test.

Treatment	LS	*g*s	E	FW	DW
	cm	*mmol* m^{-2} s^{-1}	*mmol* m^{-2} s^{-1}	*g*	*g*
Wild type	15.1 (±1.90)[a]	160 (±0.02)[a]	3.3 (±0.36)[a]	3.6 (±0.37)[a]	0.63 (±0.03)[a]
Mutant	3.7 (±0.54)[b]	96 (±0.008)[b]	2.1 (±0.17)[b]	7.0 (±0.42)[b]	0.75 (±0.04)[b]
Agar	0.1 (±0.04)[c]	80 (±0.005)[b]	1.8 (±0.11)[b]	6.6 (±0.3)[b]	0.77 (±0.04)[b]

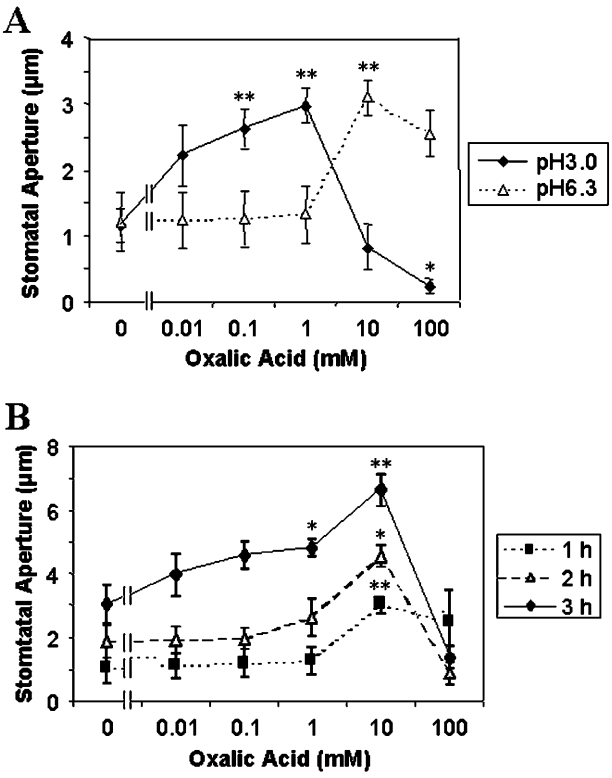

Figure 4. Dependence of stomatal aperture on oxalate concentration, pH, and exposure time. Abaxial epidermis of *V. faba* leaves was incubated with different concentrations of oxalic acid, starting with closed stomata; 0 = bath solution (45 mM KCl, 5 mM KOH, and 10 mM MES), which was used for oxalate dilutions. A, Treatment at different pH for 2 h. B, Treatment at pH 6.1 for different periods over time. Means of three independent experiments (±SE, $n = 150$) are shown; *t* tests indicate significant differences from controls at $P < 0.05$ (*) and $P < 0.01$ (**).

We tested the dicarboxylic anions oxalate, malate, malonate, and succinate for specificity of stomatal responses at 1 mM, pH 6.1. Oxalate was the only dicarboxylic anion that significantly increased stomatal aperture (Table II). Thus, guard cells specifically respond to oxalate in a concentration and pH-dependent manner.

Oxalate Stimulates K^+ Uptake and Starch Degradation in Guard Cells

To determine whether oxalate-induced changes in stomatal aperture are caused by solute accumulation, we measured potassium uptake and starch degradation in guard cells of detached abaxial leaf epidermis. Oxalic acid treatment significantly increased uptake of K^+ into guard cells compared to the buffer control (Fig. 5, A, B, and D). The staining pattern after oxalate or fusicoccin treatment was similar (Fig. 5, B and C). However, the amount of K^+ uptake into guard cells was significantly lower in the case of oxalate-treated epidermis compared to fusicoccin (Fig. 5D). Because oxalate may alter the content of organic solutes in guard cells, we also measured starch content. Oxalate significantly decreased the amount of starch in guard cell chloroplasts (Fig. 5, E–G).

Because K^+ uptake and starch degradation suggest alterations in osmotic pressure, we isolated guard cell protoplasts and exposed them to 10 mM oxalic acid. The volume of these protoplasts increased significantly in the presence of oxalate (Fig. 6, A and B), suggesting that increases in cellular solutes are responsible for stomatal opening. We also observed a significant decrease in cell number (Fig. 6C) because oxalate-treated cells burst and die. Collectively, these results suggest that an increase in osmotically active solutes is responsible for oxalate-dependent stomatal opening.

Interaction between Oxalate and ABA: Its Effect on Stomatal Control and Susceptibility to *S. sclerotiorum*

Because oxalate produced by *S. sclerotiorum* interferes with stomatal closing at night, we reasoned that oxalate may also prevent ABA-induced stomatal closure. To test this hypothesis, we challenged the abaxial epidermis of *V. faba* with different concentrations of oxalate in the presence or absence of 100 µM ABA. The aperture of light-adapted stomata decreased in response to ABA (Fig. 7). This ABA-induced decrease in stomatal aperture was significantly reduced by cotreatment with 1 and 10 mM oxalate. Oxalate significantly increased stomatal aperture compared to the buffer control at a concentration of 10 mM (paired *t* test; $P < 0.001$), but not at 1 mM (paired *t* test; $P = 0.87$).

If stomatal aperture plays a significant role in *S. sclerotiorum* virulence, mutants deficient in stomatal closure and ABA signaling would be expected to have increased susceptibility to fungal infection. We used the ABA-insensitive mutants *abi1* and *abi2*, which are impaired in stomatal closure, causing increased leaf transpiration and wilting (Leung et al., 1994, 1997). Each of these mutants has a defect in a different, but homologous, protein phosphatase 2C that acts downstream of ABA perception (Leung et al., 1994, 1997; Murata et al., 2001). The mutants *abi3* and *abi4* have defects in ABA-dependent transcriptional activators without known effects on guard cell regulation (Koornneef et al., 1984; Soderman et al., 2000). The

Table II. *Stomatal apertures (µm) after treatment with buffer or dicarboxylic anions*

The abaxial epidermis of *V. faba* was incubated in a solution containing 45 mM KCl, 5 mM KOH, 10 mM MES, pH 6.1, with or without dicarboxylic anions (1 mM) and exposed to a light intensity of 34 µmol m^{-2} s^{-1}. Means ± SEM of four separate experiments are shown. The asterisk indicates significant difference from the control at $P < 0.05$ after *t* tests.

Treatment	1 h	2 h	3 h
Buffer control	3.04 ± 0.54	4.43 ± 0.12	4.34 ± 0.61
Malate	2.69 ± 0.92	3.64 ± 0.41	4.24 ± 0.93
Succinate	3.25 ± 0.69	5.17 ± 0.60	3.82 ± 0.56
Malonate	3.47 ± 0.94	4.81 ± 0.44	4.51 ± 0.68
Oxalate	4.92 ± 0.87	5.92 ± 0.47*	6.07 ± 0.67

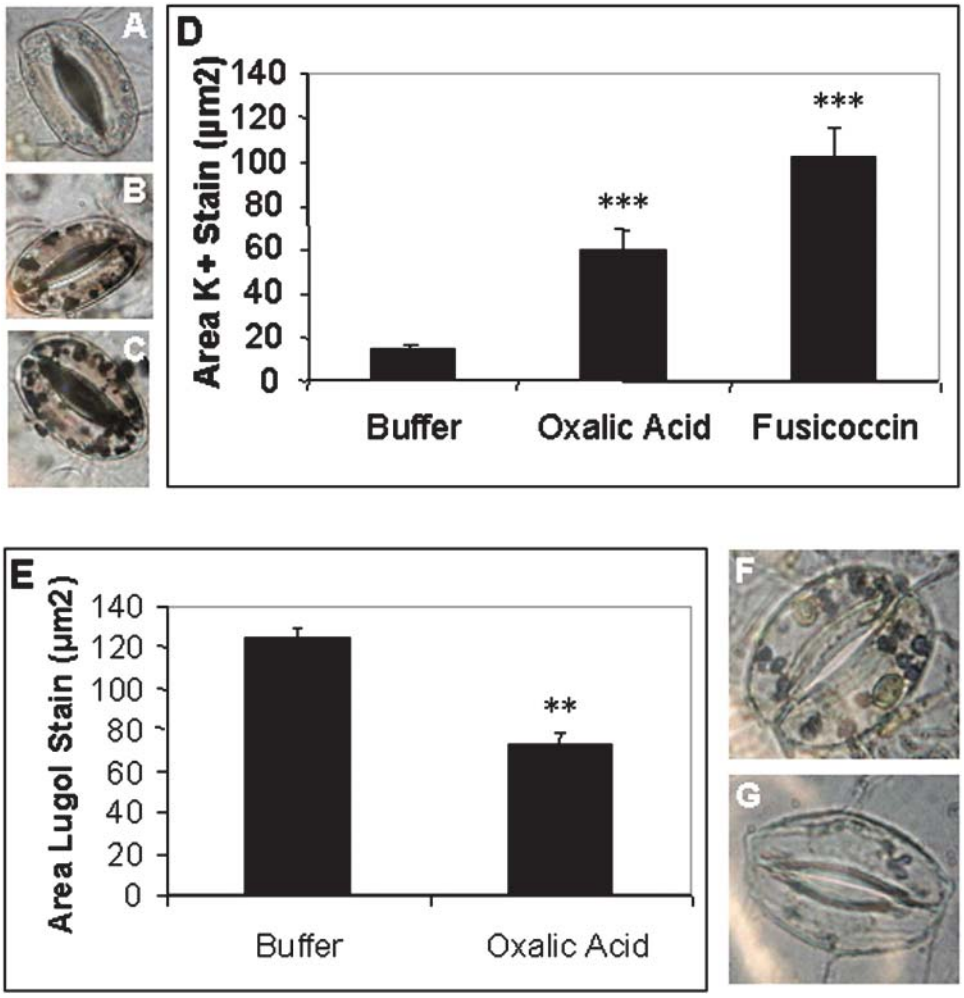

Figure 5. Oxalate induces potassium ion uptake and starch degradation in guard cells. K^+ accumulation; hexanitrocobaltate staining after treatment with buffer (A), 10 mM oxalic acid (B), 1 μM fusicoccin (C), quantification of staining using image analysis (D); means of three independent experiments (±SE, $n = 50$). This procedure precipitates all of the K^+ in the cell, regardless of its compartmental origin. Because acetic acid is used for fixation, all guard cell pairs are similar in size, regardless of treatment. Starch content; quantification of Lugol staining (E); means of three independent experiments (±SE, $n = 50$); stained cells after treatment with buffer control (F), 10 mM oxalic acid (G); *t* tests indicate significant differences from controls at $P < 0.01$ (**) and $P < 0.001$ (***).

aba2 mutant is impaired in ABA biosynthesis and stomatal regulation and sensitive to water stress (Schwartz et al., 1997; Merlot et al., 2002). We challenged these mutants and their wild-type backgrounds with the oxalate-deficient mutant of *S. sclerotiorum* to avoid the confounding influence of oxalate on disease development. The *abi4* and *aba2* mutants were significantly more susceptible to fungal attack than wild-type Arabidopsis (*Arabidopsis thaliana*) L. Heynh. ecotype Columbia (Col-0; Fig. 8A). The *abi1* and *abi3* mutants were more susceptible to fungal infection than wild-type Arabidopsis ecotype Landsberg (L*er*-0; Fig. 8B). However, the *abi2* mutant was not significantly more susceptible than its corresponding ecotype L*er*. Collectively, these results suggest that ABA contributes to resistance against *S. sclerotiorum* apparently by antagonistic interaction with oxalate.

DISCUSSION

In this article, we present evidence for guard cell dysfunction during infection of *V. faba* with *S. sclerotiorum*. This pathogen inhibits closure of stomatal pores in the dark via an oxalic acid-dependent mechanism because this cellular response was partially suppressed when plants were exposed to an oxalate-deficient fungal mutant. We confirmed oxalate deficiency by measuring oxalate production in leaves of infected *V. faba* plants. Oxalate concentrations were 25 times higher in leaves infected with wild-type *S. sclerotiorum* than in leaves challenged with the oxalate-deficient mutant 2 dpi. Whereas the mutant fungus did not significantly increase foliar oxalate levels, oxalate concentrations of approximately 10 mM were measured 2 dpi when leaves were challenged with wild-type *S. sclerotiorum*. Accumulation of oxalate in infected tissues, therefore, reaches concentrations (de Bary, 1886; Ferrar and Walker, 1993) that are high enough to induce stomatal opening in vitro. Supplementation of the fungal growth medium with succinate, which is an inducer of oxalate production, enhanced lesion expansion, albeit insignificantly (Supplemental Fig. 2). This marginal increase in pathogenicity is consistent with other small, but significant, effects of succinate on virulence (Godoy et al., 1990; T.J. Chipps, B. Gilmore, J.R. Myers, and H.U. Stotz, unpublished data). Oxalate-deficient mutants, grown in the

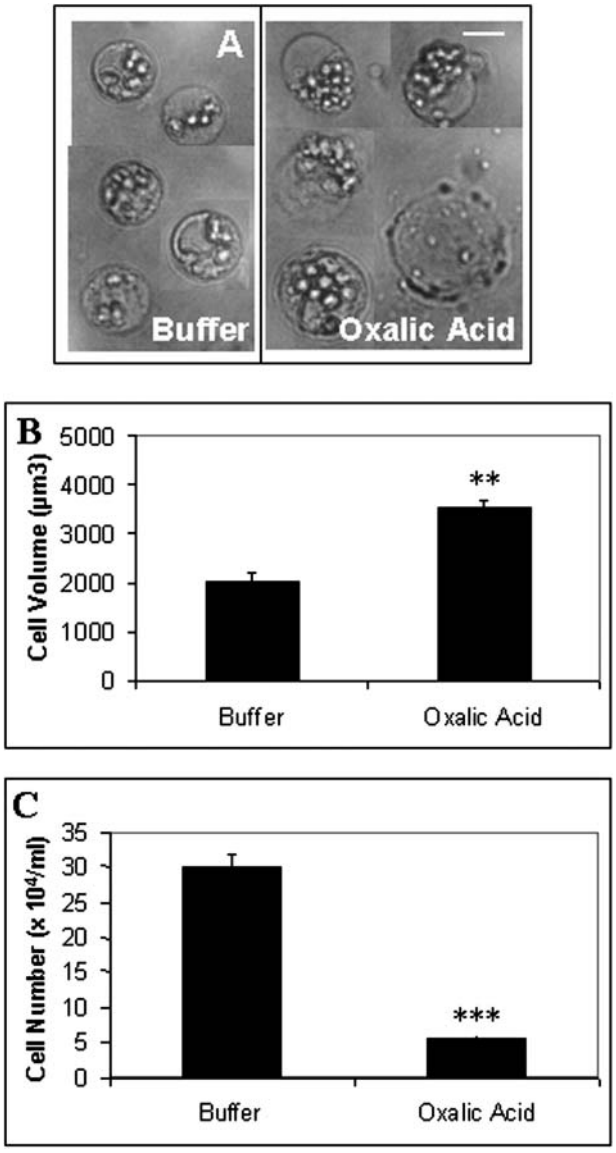

Figure 6. Oxalate induces changes in guard cell osmotic pressure. A, Micrograph of guard cell protoplasts after treatment with buffer or 10 mM oxalic acid. Bar = 10 μm. B, Protoplast volume after treatment with buffer or 10 mM oxalic acid. C, Cell number of protoplasts treated with buffer or 10 mM oxalic acid; *t* tests indicate differences from controls at $P < 0.01$ (**) and $P < 0.001$ (***).

presence of succinate, still produce 5 times less oxalate than wild-type *S. sclerotiorum* (Godoy et al., 1990). This lower level of oxalate production is perhaps insufficient for the mutant to cause a dramatic shift in pathogenicity (Godoy et al., 1990; T.J. Chipps, B. Gilmore, J.R. Myers, and H.U. Stotz, unpublished data). Oxalate-deficient mutants grow 19% to 28% slower than wild-type *S. sclerotiorum* and do not produce sclerotia (Godoy et al., 1990). A reversion restored oxalate production, sclerotia formation, and growth rate, suggesting that all three phenotypes were caused by disruption of a single gene (Godoy et al., 1990). We factored out differences in growth rate when comparing stomatal apertures between wild-type and mutant *S. sclerotiorum* because we measured stomatal apertures within and outside necrotic lesions regardless of their size. Growth rate was therefore not relevant for these measurements. Thus, oxalate deficiency is most likely responsible for reduced stomatal responses to *S. sclerotiorum*, although we cannot entirely exclude the contribution of other factors because oxalate-deficient mutants have not been genetically characterized.

We detected open stomata in advance of invading hyphae, suggesting that oxalate moves faster than the mycelium. This observation supports previous data on the systemic spread of oxalate through the vascular tissue (Noyes and Hancock, 1981). *S. sclerotiorum* induces an oxalate-dependent increase in stomatal conductance and transpiration that results in a reduction of biomass. These results provide an alternative explanation for oxalate-induced wilting. Previously, oxalate crystal formation and embolisms in the xylem were suspected to cause water stress (Lumsden, 1979; Sperry and Tyree, 1988). Exposure to *S. sclerotiorum* causes a decrease in dry weight, which indirectly suggests that the fungus metabolizes host-derived carbohydrates.

In addition to causing water stress, open stomata were exploited for hyphal emergence and secondary colonization. We confirmed that initial penetration of *V. faba* leaves occurred via infection cushions (Prior and Owen, 1963; Lumsden and Dow, 1973; Lumsden and Wergin, 1980; Jamaux et al., 1995) as early as 9 h postinoculation (hpi; Supplemental Fig. 3). We have not studied the formation of penetration pegs beneath these infection cushions, but it is known that Sclerotinia penetrates through the cuticle using enzymes and/or mechanical force (Prior and Owen, 1963; Lumsden and Dow, 1973; Lumsden and Wergin, 1980) or through stomatal pores (Prior and Owen, 1963; Jones, 1976). In agreement with published reports (Lumsden and Dow, 1973; Lumsden and Wergin, 1980), we observed ramifying hyphae of Sclerotinia emerge

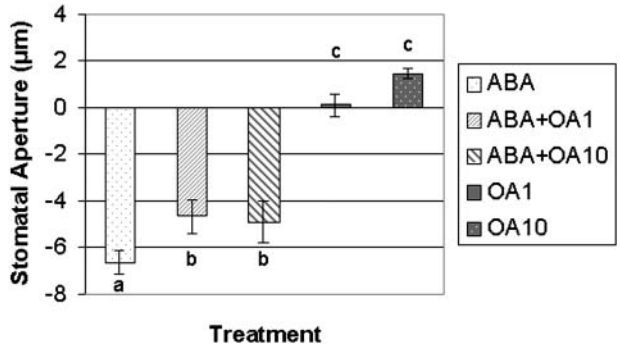

Figure 7. Changes in stomatal aperture in response to ABA and oxalic acid (OA). The abaxial epidermis was incubated in buffer (45 mM KCl, 5 mM KOH, and 10 mM MES, pH 6.1) in the presence or absence of ABA, oxalic acid, or cotreatments, starting with opened stomata. Means of five independent experiments (±SE, $n = 250$) are shown. Stomatal apertures were measured 1 h after the onset of treatment. Letters a, b, and c indicate significant differences ($P < 0.05$) among treatments according to ANOVA and Duncan's Multiple Range Test.

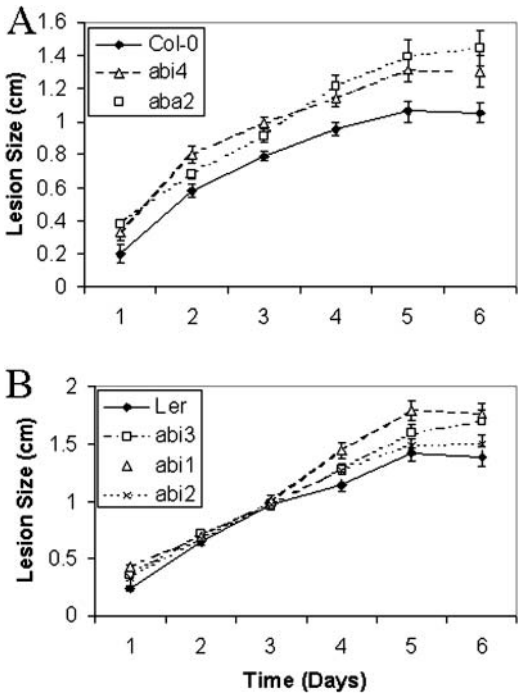

Figure 8. Disease progression of the oxalate-deficient *S. sclerotiorum* mutant on Arabidopsis. The repeated-measures procedure of a general linear model was used for statistical analysis. A, Lesion size after foliar infection of *abi4-1* and *aba2-1* mutant and wild-type Arabidopsis Col-0. Mutants were significantly more susceptible than wild-type plants (LSD; $P < 0.001$). B, Lesion sizes after foliar infection of *abi1-1*, *abi2-1*, and *abi3-1* mutant and wild-type Arabidopsis L*er*-0. The *abi1-1* and *abi3-1* mutants (LSD; $P < 0.001$), but not the *abi2-1* mutant (LSD; $P = 0.055$), were more susceptible than wild-type plants. Means ±SE are shown from a minimum of six plants. Experiments were repeated twice with similar results.

through stomata from the inside of infected leaves. Hyphal emergence results in secondary colonization and formation of sclerotia on the host surface (Lumsden and Dow, 1973; Lumsden and Wergin, 1980). Stomatal opening therefore facilitates hyphal movement out of infected host tissues.

To understand the mechanisms of oxalate-dependent guard cell dysfunction, we studied stomatal responses in the detached abaxial epidermis of *V. faba*. Oxalate induces stomatal opening at concentrations between 1 and 10 mM. Because these concentrations are frequently exceeded in infected tissues (de Bary, 1886; Godoy et al., 1990; Ferrar and Walker, 1993), oxalate is active at physiologically relevant concentrations in vitro. Stomatal opening is also specific to oxalic acid because other dicarboxylic acids do not cause this cellular response. Transport of oxalate across the plasma membrane (Kostman et al., 2001) perhaps accounts for the fact that incubation of the epidermis at decreasing pH causes stomatal opening and cell death to occur at lower oxalate concentrations.

Oxalate increased K^+ uptake and starch degradation in guard cells. Potassium channels are known to be involved with osmosensing and turgor regulation of guard cells (Liu and Luan, 1998). Sugars from photosynthesis and starch degradation provide additional osmotica for the regulation of guard cell turgor (Tallman and Zeiger, 1988). Blue light, like oxalate, induces starch loss in guard cell chloroplasts as well as K^+ uptake (Tallman and Zeiger, 1988). Both of these processes are expected to increase guard cell osmotic pressure, which was confirmed because oxalate induced protoplast swelling. In addition to oxalate, endo-polygalacturonase and pectin methylesterase are expected to cause open stomatal pores during Sclerotinia infection (Riou et al., 1992; Jones et al., 2003). Synergism between oxalate- and pectin-degrading enzymes has been previously suggested because oxalate decreases the pH of infected tissues to stimulate their activity (Bateman and Beer, 1965). Moreover, oxalate is expected to increase substrate availability of endo-polygalacturonase by removing calcium ions bound to pectin (Bateman and Beer, 1965). This would probably explain why we found partially open stomatal pores in plants infected with the oxalate-deficient mutant of *S. sclerotiorum*.

Because secretion of oxalate by *S. sclerotiorum* prevents stomatal closure in the dark, we tested whether oxalate interferes with ABA-induced stomatal closing. Simultaneous exposure of the abaxial epidermis of *V. faba* to oxalate and ABA inhibited stomatal closing relative to treatment with ABA alone. At present, we do not know the mechanism by which oxalate suppresses ABA-induced stomatal closure. Oxalate may simply oppose ABA action by replenishing guard cells with K^+. Alternatively, oxalate may modulate signal transduction processes. ABA is known to activate plasma membrane-localized Ca^{2+} and anion channels, leading to inactivation and activation of inward- and outward-rectifying K^+ channels, respectively (Schroeder et al., 1987; Keller et al., 1989; Schroeder and Hagiwara, 1989, 1990). H_2O_2 is a signaling intermediate that acts downstream of ABA and upstream of Ca^{2+} channels (Pei et al., 2000). H_2O_2 appears to be a point of conversion for ABA and pathogenic elicitors (Klusener et al., 2002). Oxalate, on the other hand, suppresses elicitor-stimulated H_2O_2 production in tobacco and soybean cells (Cessna et al., 2000).

The Arabidopsis mutants *abi1*, *abi3*, *abi4*, and *aba2*, which have defects in ABA sensing or ABA biosynthesis, were consistently more susceptible to oxalate-deficient *S. sclerotiorum* than wild-type plants. Because *abi1* and *aba2* mutants are impaired in guard cell regulation, stomatal opening is apparently required for optimal fungal colonization. In contrast to the *abi1* mutant, increased susceptibility of the *abi2* mutant to *S. sclerotiorum* was insignificant. This phenotypic difference may be due to the stronger ABA insensitivity of *abi1* mutants relative to *abi2* mutants (Leung et al., 1997). The increased susceptibility of the *abi3* and *abi4* mutants to *S. sclerotiorum* is more difficult to reconcile because these mutations were classified as specific for seed germination (Koornneef et al., 1998). However, the *abi4* mutation was recently shown to interfere with

β-amino-butyric acid-induced resistance against the necrotrophic pathogen *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004). This recent report supports our conclusion that ABA biosynthesis and signaling contribute to resistance against *S. sclerotiorum*.

Regulation of stomatal movement involves a myriad of signals in response to an array of environmental and physiological cues (Hetherington and Woodward, 2003). Our results provide, for the first time to our knowledge, compelling evidence that oxalate impacts guard cell function in response to a fungal pathogen.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) L. Heynh. mutant seeds *aba2-1* (CS156), *abi1-1* (CS22), *abi2-1* (CS23), *abi3-1* (CS24), and *abi4-1* (CS3836) and ecotypes Col-0 and L*er*-0 were obtained from The Arabidopsis Information Resource (TAIR). *Vicia faba* L. cv Broad Windsor seeds were purchased from Territorial Seed (Cottage Grove, OR). *V. faba* and Arabidopsis were cultivated from seeds under controlled environmental conditions. *V. faba* was grown with an 8-h photoperiod of white light (300 μmol m^{-2} s^{-1}) at 25°C. Arabidopsis was grown with a 12-h photoperiod of white light (200 μmol m^{-2} s^{-1}) at 25°C.

Fungal Growth and Plant Inoculations

A wild-type isolate (1980) and an oxalate-deficient mutant (A4) of *Sclerotinia sclerotiorum* (Lib.) de Bary were kindly provided by Dr. Martin Dickman (University of Nebraska, Lincoln, NE) and grown on potato dextrose agar as described by Godoy et al. (1990). Potato dextrose agar was supplemented with 1.5% (w/v) sodium succinate to test recovery of pathogenicity of oxalate-deficient *S. sclerotiorum*. An agar plug (0.4 mm in diameter) containing the advancing edge of growing mycelia was removed and used to inoculate the adaxial surface of *V. faba* or Arabidopsis leaves. Three leaves per Arabidopsis plant were inoculated with the oxalate-deficient mutant using one plug per leaf. Infected plants were kept in a clear plastic box under saturating humidity and a 12-h light photoperiod at 20°C and a light intensity of 34 μmol m^{-2} s^{-1} using fluorescent white lights. Lesion diameters were measured with a caliper on a daily basis for up to 6 d.

Physiological Measurements

To test effects on physiology of *V. faba* plants, we inoculated wounded stems of 1-month-old plants with agar containing wild-type or mutant *S. sclerotiorum* or agar alone (Petzholdt and Dickson, 1996). We analyzed stomatal conductance and transpiration 4 dpi using a LI-COR LI-1600 porometer (Lincoln, NE). Measurements were performed on three nonwilting pairs of leaves at nodes below the inoculation site. Lesion size was measured with a caliper and infected stems were collected for fresh weight and dry weight analyses 12 d after infection; stems were dried at 60°C in an oven for 3 d.

Oxalate Concentration Measurement

Leaves of *V. faba* plants were inoculated and incubated for up to 2 d with agar plugs containing *S. sclerotiorum* mycelia. Control leaves were mock inoculated with agar plugs and incubated for 2 d. Treated or control leaves (less than 0.8 g of fresh weight) were homogenized in 3.5 mL of 0.2 M potassium phosphate, pH 6.5 (Ferrar and Walker, 1993), using a Polytron homogenizer. Oxalate concentrations of the extracts were measured colorimetrically using an enzymatic method (procedure 591; Sigma, St. Louis). Oxalate standards (0.25 mM, 0.5 mM, 0.75 mM, 1 mM, and 2.5 mM) were used for quantification.

Stomatal Aperture Measurements

The abaxial epidermis was peeled from the youngest fully expanded leaves of 1-month-old plants of *V. faba*. Epidermis was floated on a bath solution containing 45 mM KCl, 5 mM KOH, and 10 mM MES, pH 6.1, with or without treatment; pH was adjusted with NaOH. Opening experiments were initiated with closed stomata from leaves kept in darkness. Closure experiments were initiated with open stomata from leaves kept in white light (34 μmol m^{-2} s^{-1}) at 20°C for 2 h. Epidermis was incubated in the bath solution for 1 h prior to treatment. Width of the innermost cell wall of guard cells was determined with an ocular micrometer and a Leica (Wetzlar, Germany) DME light microscope.

Fluorescence Microscopy

A GFP-tagged strain of *S. sclerotiorum* (Lorang et al., 2001) was used for fluorescence microscopy. Plants were kept in the dark for at least 6 h prior to the experiment. Inoculated *V. faba* leaf sections were mounted on a slide with water and visualized with a Leica TCS 4D laser scanning confocal microscope equipped with an Omnichrome Ar/Kr Laser, excitation at 488/568 nm, and emission at 590 nm, long-pass and fluorescein isothiocyanate band-pass filters, and an INNOVA Enterprise Ion Laser (Coherrent, Santa Clara, CA). Optical sections were processed using Image Pro Plus (Carlsbad, CA). Alternatively, a Leica MZ FLIII stereomicroscope equipped with a filter set that matches the spectral properties of GFP was used. Specifically, the GFP Plus fluorescence filter set was used. The excitation filter had the following characteristics: 480/40 nm. The barrier filter transmitted emission wavelengths larger than 510 nm.

Fluorescein diacetate was used at a concentration of 0.01% to assess cell viability as recommended previously (Poffenroth et al., 1992).

Analysis of Starch and K^+

Epidermis was treated with or without 10 mM oxalic acid in bath solution containing 45 mM KCl, 5 mM KOH, and 10 mM MES, pH 6.1, for 2 h at 20°C in the dark, washed in water, and stained with 10% Lugol's iodine solution (ICN, Costa Mesa, CA) for 10 min. Epidermis was briefly rinsed with water prior to microscopy and image analysis. Cell walls were erased from guard cell images using Photoshop (Microsoft, Seattle) and the stained areas of chloroplasts were assessed. All images were converted to 8-bit using Image Pro Plus and analyzed using a range of 0 and 90. Cumulative data were exported and analyzed using Excel (Microsoft).

Sodium hexanitrocobaltate staining was used to measure K^+ content; fresh solutions were prepared prior to each experiment (Green et al., 1990). Epidermis was incubated in a 1:10 dilution of bath solution with or without 10 mM oxalic acid for 1.5 h at 20°C in the dark. After three rinses in water, epidermis was incubated in sodium hexanitrocobaltate (0.5 M) dissolved in 10% (v/v) acetic acid for 10 min. Epidermis was washed in water and mounted on a slide containing a drop of 5% (v/v) ammonium sulfide. Image analysis was performed as described above. Digital images were converted to 8-bit using Image Pro Plus and analyzed using a threshold of 0 and 90. Cumulative areas of CoS granules were calculated per guard cell pair. Stained areas are known to be proportional to stomatal apertures (Green et al., 1990).

Guard Cell Protoplasts

To measure changes in osmotic pressure, we isolated guard cell protoplasts from *V. faba* according to Pandey et al. (2002). Protoplasts were incubated in a solution of 10 mM oxalic acid in basic medium (100 μM KH_2PO_4, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ascorbic acid, 450 mM sorbitol, and 5 mM MES, pH 5.5, adjusted with KOH) for 30 min at 20°C and then viewed under a light microscope to determine cell size with an ocular micrometer.

Statistical Analysis

Statistical tests included ANOVA, using the SAS program package (Cary, NC). Differences in stomatal conductance, transpiration, lesion size, fresh weight, and dry weight between treatments were compared using Duncan's Multiple Range Test. Student's *t* tests were used to compare treatment and control means for experiments involving detached epidermis. Experiments with Arabidopsis mutants and ecotypes were compared using the repeated-measures procedure of a general linear model; means were compared using LSD.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Jim Myers for assisting with statistical analysis and critical review of the manuscript; thanks also to Dr. Gary Tallman and Dr. Lawrence Talbott for valuable suggestions.

Received July 13, 2004; returned for revision August 23, 2004; accepted August 24, 2004.

LITERATURE CITED

Bateman DF, Beer SV (1965) Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis of *Sclerotium rolfsii*. Phytopathology **55:** 204–211

Burke JM, Riesenberg LH (2003) Fitness effect of transgenic disease resistance in sunflowers. Science **300:** 1250

Cessna SG, Sears VE, Dickman MB, Low PS (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. Plant Cell **12:** 2191–2199

de Bary A (1886) Ueber einige Sclerotinien and Sclerotienkrankheiten. Botanische Zeitung **44:** 377–474

Donaldson PA, Anderson T, Lane BG, Davidson AL, Simmonds DH (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. Physiol Mol Plant Pathol **59:** 297–307

Ferrar PH, Walker JRL (1993) o-Diphenol oxidase inhibition—an additional role for oxalic acid in the phytopathogenic arsenal of *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. Physiol Mol Plant Pathol **43:** 415–422

Godoy G, Steadman JR, Dickman MB, Dam R (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiol Mol Plant Pathol **37:** 179–191

Green DB, Dodge SM, Lee JR, Tallman G (1990) Effect of sodium hexanitrocobaltate-III decomposition on its staining of intracellular potassium ions. Stain Technol **65:** 15–24

Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. Nature **424:** 901–908

Holmes RP, Assimos DG (1998) Glyoxylate synthesis, and its modulation and influence on oxalate synthesis. J Urol **160:** 1617–1624

Hu X, Bidney DL, Yalpani N, Duvick JP, Crasta O, Folkerts O, Lu G (2003) Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. Plant Physiol **133:** 170–181

Jamaux I, Gelie B, Lamarque C (1995) Early stages of infection of rapeseed petals and leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. Plant Pathol **44:** 22–30

Jones D (1976) Infection of plant tissue by *Sclerotinia sclerotiorum*: a scanning electron microscope study. Micron **7:** 275–279

Jones L, Milne JL, Ashford D, McQueen-Mason SJ (2003) Cell wall arabinan is essential for guard cell function. Proc Natl Acad Sci USA **100:** 11783–11788

Keller BU, Hedrich R, Raschke K (1989) Voltage-dependent anion channels in the plasma membrane of guard cells. Nature **341:** 450–453

Kesarwani M, Azam M, Natarajan K, Mehta A, Datta A (2000) Oxalate decarboxylase from *Collybia velutipes*: molecular cloning and its overexpression to confer resistance to fungal infection in transgenic tobacco and tomato. J Biol Chem **275:** 7230–7238

Klusener B, Young J, Murata Y, Allen GJ, Mori IC, Hugouvieux V, Schroeder J (2002) Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. Plant Physiol **130:** 2152–2163

Kolkman JM, Kelly JD (2000) An indirect test using oxalate to determine physiological resistance to white mold in common bean. Crop Sci **40:** 281–285

Koornneef M, Leon-Kloosterziel KM, Schwartz SH, Zeevaart JAD (1998) The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in Arabidopsis. Plant Physiol Biochem **36:** 83–89

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol Plant **61:** 377–383

Kostman TA, Tarlyn NM, Loewus FA, Franceschi VR (2001) Biosynthesis of L-ascorbic acid and conversion of carbons 1 and 2 of L-ascorbic acid to oxalic acid occur within individual calcium oxalate crystal idioblasts. Plant Physiol **125:** 634–640

Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J (1994) Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science **264:** 1448–1452

Leung J, Merlot S, Giraudat J (1997) The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9:** 759–771

Liu K, Luan S (1998) Voltage-dependent K^+ channels as targets of osmosensing in guard cells. Plant Cell **10:** 1957–1970

Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, Rollins JA, Wolpert TJ, Johnson KB, Rodriguez RJ, Dickman MB, et al (2001) Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol **67:** 1987–1994

Lumsden RD (1979) Histology and physiology of pathogenesis in plant diseases caused by *Sclerotinia* species. Phytopathology **69:** 890–896

Lumsden RD, Dow RL (1973) Histopathology of *Sclerotinia sclerotiorum* infection of bean. Phytopathology **63:** 708–715

Lumsden RD, Wergin WP (1980) Scanning-electron microscopy of infection of bean by species of *Sclerotinia*. Mycologia **72:** 1200–1209

Merlot S, Mustilli AC, Genty B, North H, Lefebvre V, Sotta B, Vavasseur A, Giraudat J (2002) Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. Plant J **30:** 601–609

Murata Y, Pei Z-M, Mori IC, Schroeder J (2001) Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. Plant Cell **13:** 2513–2523

Nakagawa Y, Shimazu K, Ebihara M, Nakagawa K (1999) *Aspergillus niger* pneumonia with fatal pulmonary oxalosis. J Infect Chemother **5:** 97–100

Noyes RD, Hancock JG (1981) Role of oxalic acid in the *Sclerotinia sclerotiorum* wilt of sunflower *Helianthus annuus*. Physiol Plant Pathol **18:** 123–132

Pandey S, Wang XQ, Coursol SA, Assmann SM (2002) Preparation and applications of *Arabidopsis thaliana* guard cell protoplasts. New Phytol **153:** 517–526

Pei Z-M, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406:** 731–734

Petzholdt R, Dickson M (1996) Straw test for resistance to white mold in bean. Bean Improv Cooperative Annu Rep **39:** 142–143

Poffenroth M, Green DB, Tallman G (1992) Sugar concentrations in guard cells of *Vicia faba* illuminated with red or blue light. Analysis of high performance liquid chromatography. Plant Physiol **98:** 1460–1471

Prior GD, Owen JH (1963) Pathological anatomy of *Sclerotinia trifolium* on clover and alfalfa. Phytopathology **54:** 784–787

Riou C, Freyssinet G, Fevre M (1992) Purification and characterization of extracellular pectinolytic enzymes produced by *Sclerotinia sclerotiorum*. Appl Environ Microbiol **58:** 578–583

Schroeder J, Hagiwara S (1990) Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of nonselective Ca^{2+} permeable channels. Proc Natl Acad Sci USA **87:** 9305–9309

Schroeder JI, Hagiwara S (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. Nature **338:** 427–430

Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K^+ channels in guard-cell protoplasts. Proc Natl Acad Sci USA **84:** 4108–4112

Schwartz SH, Leon-Kloosterziel KM, Koornneef M, Zeevaart JAD (1997) Biochemical characterization of the aba2 and aba3 mutants in *Arabidopsis thaliana*. Plant Physiol **114:** 161–166

Soderman EM, Brocard IM, Lynch TJ, Finkelstein RR (2000) Regulation and function of the Arabidopsis ABA-insensitive4 gene in seed and abscisic acid response signaling networks. Plant Physiol **124:** 1752–1765

Sperry JS, Tyree MT (1988) Mechanism of water stress-induced xylem embolism. Plant Physiol **88:** 581–587

Tallman G, Zeiger E (1988) Light quality and osmoregulation in *Vicia* guard cells. Evidence for involvement of three metabolic pathways. Plant Physiol **88:** 887–895

Ton J, Mauch-Mani B (2004) (beta)-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J **38:** 119–130